Fall 2018

Investigating Vegetative Phase Change in Maize

Michael Kline

INVESTIGATING VEGETATIVE PHASE CHANGE IN MAIZE

by

Michael Kline

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Biology

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Erin Irish
Thesis Mentor

Fall 2018

All requirements for graduation with Honors in the
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Lori Adams
Biology Honors Advisor

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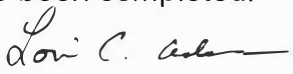

Lori Adams, PhD.
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Abstract

Vegetative phase change is a plants change from its juvenile phase to its reproductive adult phase. During this change there are abrupt changes in plant morphology and this can vary widely from plant to plant. Some plants like trees can take decades to transition into the adult phase, while other like maize and *Arabidopsis thaliana* can take only weeks. Both of these phases are regulated by miR156, which decline at the end of the juvenile phase resulting in the increase of miR172 miR395 experiences a peak in expression in early development of maize in leaves 1 and 2. There is also another peak in expression in the primordia of leaf 5, starting the transition into the adult phase. The regulation of these microRNAs are still in question. Here, we look at how synergistic effects of jasmonic acid and salicylic acid effect the regulation of vegetative phase change. We also applied a slow releasing hydrogen sulfide compound, GYY4137, and quantified photosynthetic pigments as a way to look at the role of miR395 in the juvenile phase.

Acknowledgements

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Introduction

Like many organisms, angiosperms undergo multiple stages of development. In angiosperms, the maturation that permits the ability to reproduce sexually is called vegetative phase change (Brink, 1962). A prominent transition is that from the vegetative growth to flowering (Simpson *et al*., 1999). For this to occur, the vegetative juvenile stage must transition into an adult, preceding the ability to flower (Poethig, 1990). In many species, juvenile organs change patterns of differentiation during phase change, when they become adult (Hackett, 1985). Adult organs are marked by differentiation that is distinct from that of the juvenile organs (Kerstetter and Poethig, 1998). An example of a woody species, English Ivy (*H. Helix*) has very distinct differences in juvenile and adult growth. Juvenile ivy grows horizontally on the ground and forms lobed leaves. Adult ivy plants grow vertically and has oval leaves (Hackett, 1985). The difficulty of studying English Ivy is similar to many other woody species, which is its prolonged juvenile phase. Ivy can be in the juvenile phase for at least a decade. Other woody species, such as popular trees can have the juvenile phase persist for thirty to forty years. In annual species, such as rice, maize, and *Arabidopsis thaliana*, phase change is relatively short making them good organisms to study phase change.

In wild type maize, the shoot apical meristem initiates a determined number of leaves (approx. 16 to 22) before a terminal inflorescence: the tassel. The first four to five leaves are juvenile (Poethig 1990). Phase change occurs in the next two to three leaves. These are called transition leaves and are mosaics of juvenile and adult traits (Bongard-Pierce *et al*., 1996). Leaves initiated after phase change are entirely adult (Poethig, 1990). Morphological and histochemical differences in the epidermis show the variation in form and function in a fully

expanded leaf blades (Bongard-Pierce *et al.*, 1996; Evans *et al.*, 1994; Freeling and Lane, 1994; Moose and Sisco, 1994; Poethig, 1990). The surface of juvenile leaves lack trichomes (thin hairs on epidermis of leaves) and has epicuticular wax causing a dull blue-green color. Trichomes are a feature of adult leaves, and adult leaves lack epicuticular wax. Epidermal cell walls of a juvenile leaf are weakly crenulated and stain violet with Toluidine Blue-O due to a lack of lignin with the cell walls. Adult epidermal cells are strongly crenulated and are aquamarine when stained with Toluidine Blue-O, indicating the presence of lignin.

On a molecular level, highly conserved microRNAs (miRNAs), miR156 and miR172, have been shown to promote the transition from juvenile to adult and to flowering (Schwartz *et al*., 2008; Wu and Poethig, 2006). miR156 targets *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factors that up regulates miR172 (Klein *et al*., 1996). miR172 targets mRNAs that encode two APETALA 2 (AP2) DNA-binding domains. These proteins have been shown to regulate the transition to flowering and flower development (Aukerman and Sakai, 2003; Mathieu *et al.*, 2009; Schmid *et al.*, 2003).

Mutants overexpressing the miR156 gene show a prolonged juvenile phase, increased branching, accelerated leaf production, and delayed flowering in Arabidopsis due to miR156 blocking miR172 by degrading SPL (Wu and Poethig, 2006) and in maize (Chuck et al., 2007). Loss of function mutations result in the opposite phenotype: slow growing, fewer leaves and flowers, and early flowering (Yang et al., 2013).

While miR156 and 172 regulate the genes that result in phase-specific patterns of differentiation, it is not known what causes miR156 to be high at the beginning of the juvenile phase (at seed germination). Research in the Irish lab at the University of Iowa has investigated this by comparing gene expression patterns in leaf primordia that will become either juvenile or

adult leaves. An unexpected finding was the high expression in early juvenile leaves of genes that result in the formation of the hormones jasmonic acid (JA) and salicylic acid (SA) (Beydler et al, 2016). These hormones are known to be associated with responses to stress. Those studies also showed that early juvenile leaves experience stress from ROS associated with the onset of growth in light. This prompted the hypothesis that juvenility may be the result of stress status in the seedling, mediated by the hormones JA and SA. This hypothesis was tested by application of either JA or SA to seedlings. In the case of JA, a delay in phase change was observed, including a prolonged expression of miR156. Treatment with SA did not have a significant effect (Beydler et al, 2016).

Of the genes highly expressed in the juvenile state, approximately 30% of them were involved with photosynthesis (Strable *et al*., 2008). The next highest set of genes that were upregulated in juvenile plants were genes for oxidation reduction (Beydler, 2014). This might be explained by the generation of reactive oxygen species (ROS) by photosynthetic elements (Asada, 2006). ROS, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide ($O_2\cdot^-$), and hydroxyl radicals (HO·), are toxic molecules causing oxidative damage to DNA, lipids and proteins. ROS is generated during environmental stresses coming from changes in light, temperature, salinity, drought, nutrient deficiency, and pathogen attack. Although these are common events in the environment, plants have an array of anti-oxidants and anti-oxidative enzymes that dissipate ROS. It is when there is an imbalance of ROS and these detoxification enzymes and molecules that oxidative stress and damage occurs. If severe enough, oxidative damage can lead to cell death. ROS also act as signaling molecules involved in growth and developmental processes, pathogen defense response, stress hormone production, and programmed cell death (Apel and Hirt, 2004).

The stress hormone JA is known to have mediating roles in responses to biotic and abiotic stressors including oxidative damages (Creelman and Mullet, 1997). Our lab has shown JA is high in juvenile leaves, and lower in adult (Beydler *et al*., 2016). Furthermore, our lab has hypothesized that JA promotes the juvenile vegetative phase in response to stresses involved in seedling development. Therefore, once a seedling is able to relieve the oxidative stress, the following JA level decline mediates the decline in miR156 allowing the plant to transition into an adult phase with reproductive ability (Beydler *et al*., 2016).

Preliminary results suggest that sulfur may have a role as a signaling molecule in the juvenile phase. Sulfur is an essential macronutrient required for normal plant growth. Typically, sulfate enters the plant through the soil using sulfate transporters in the root hairs and travels to the leaves via the xylem (Leustek *et al*., 2000). In the leaves, sulfate is reduced to sulfide and then assimilated into cysteine (Leustek *et al*., 2000), a precursor to methionine, S-adenosylmethionine, glutathione (GSH), and glucosinolates **(**Rausch and Wachter, 2005). Cysteine and methionine are essential amino acids derived from sulfur for protein synthesis.

In addition to sulfur's role as a nutrient, it has been recognized as a component of a signaling molecule, hydrogen sulfide (H_2S) (Hancock et al., 2011; García-Mata and Lamattina, 2010; Lisjak et al., 2011). Although there have been few experiments studying hydrogen sulfide as a signaling molecule in plants, it has been observed that H_2S is lethal to plants at high concentrations (Chen et al., 2011; Oren and Padan, 1979; Cohen et al., 1986; Dooley et al., 2013). H_2S at low concentrations decreases time to germination (Zhang et al., 2008). Other studies have shown H_2S increases resistance to drought and heavy metal toxicity (Zhang et al., 2008**;** Zhang et al., 2010; Wang et al., 2012; Thompson and Kats, 1978). Finally, exposure to

low levels of H_2S has increased chloroplast biogenesis (Chen et al., 2011) and has a signaling role in guard cells (Hancock et al., 2011; García-Mata and Lamattina, 2010; Lisjak et al., 2011)

Our lab observed that miR395 is upregulated early in the development of leaves 1, 2, and 5 in *Zea mays* (unpublished). miR395 targets a low-affinity sulfate transporter (SULTR) and three ATP sulfurylases (APS1, APS3 and APS4) in higher plants (Jagadeeswaran et al., 2014). These molecules are the first two steps in the sulfur assimilation pathway and when degraded by miR395, free sulfur concentration increases in plant tissue (Liang et al., 2010). In another study with *Arabidopsis*, GSH abundance declined (Kawashima *et al*., 2011; Matthewman *et al*., 2012) and miR395 increased during sulfur deprivation (Jones-Rhoades and Bartel, 2004).

First, I tested JA and SA in tandem to see if they play a synergistic role in vegetative phase change. To do this I applied SA followed by varying doses of JA and vice versa. To test the hypothesis that there is more ROS in the juvenile state that the adult we qualitatively and quantitatively examined ROS in maize leaves. To observe the location and quantity of ROS build up from developmental cues, Nitro Blue Tetrazolium (NBT) and 3,3′-Diaminobenzidine (DAB)stains was used. Knowledge of location, quantity, and type of ROS in leaves should allow us to determine if ROS is an important signal in a plant's developmental process. Furthermore, to understand the peaks in expression of miR395 in early development and the role of H_2S as a signaling molecule, we applied a slow releasing hydrogen sulfide compound (GYY4137) to *Zea mays* and quantified light harvesting molecules chlorophyll a, chlorophyll b, and carotenoids. We hypothesized that higher concentrations of hydrogen sulfide will increase photosynthetic activity because H_2S may be the signaling molecule that up-regulates photosynthetic genes early in juvenile development. Results and Discussion for each experiment will follow in separate section from each other.

Materials and Methods

Synergistic Effects of JA and SA

Seeds of maize, cv. Golden Bantam, were planted in potting mix in the greenhouse. Plants were then sorted by when they germinated to keep consistency for dosing levels. Seedlings were first treated with one hormone (5 mM JA dissolved in 5% ethanol or 15 mM SA dissolved in water), then followed by variable number of applications of the other hormone in subsequent days and vice versa. Hormone solutions were applied by pipetting the solution into the apical whorl of the seedling. Half of the seedlings received jasmonic acid treatments first (JA-SA) and the other half received salicylic acid treatments (SA-JA). After application of the initial hormone, each group was further subdivided and received 200 µL applications 0, 1, 2, 3, 4, or 5 times, every sequential day, while control plants received only the initial treatment. For the SA-JA treatments, the control received 200 µL of water and then 200 µL of 5% ethanol for the remainder of the dosing period. Meanwhile, on the first day, all the other Sa-JA plants received 200 µL of 15 mM salicylic acid. Each will receive their corresponding amount of jasmonic acid at 200 µL per day. Once the SA-JA grouping received their specific amount of JA, 5% ethanol was given to the plants so all plants received an equal number of treatments over time. Adding 5% ethanol after JA doses were completed also ensured the ethanol was not a variable skewing results.

For JA-SA treatments, the control started with a dose of 200 µL of 5% ethanol and all the other levels received 200 µL of 5 mM JA. The control received 200 µL of water for the remainder of the dosages. All other groups received their corresponding doses of SA. Water was added after the completion of the hormone dosages. Once treatments were completed, the plants

were moved to the greenhouse to grow until hairs were present on an entire leaf, marking the entry into the adult phase.

As leaves developed, leaf 5 was marked to ensure its identity for future scoring. Once the plants have grown to the desired maturity, leaves 5 through 9 were scored. Scoring entailed examining hair pattern on the leaf and staining with Toluidine Blue to observe the presence (aquamarine) or lack of lignin (purple). In the transition zone, staining shows a mosaic of purple and aquamarine. Hair patterns are classified into three categories of full hair (adult), transition, or absent (juvenile). Staining is also classified into three categories of lignin (adult), mosaic, or lack of lignin (juvenile). Wax was classified as present or absent.

Naming Scheme for Plants

Naming of plants from here on out will go as follows. SA-JA will stand for plants that received an initial dose of SA prior to JA treatments. JA-SA will stand for plants that received an initial dose of JA followed by SA doses. To determine how many doses each plant received of the second hormone, it will be labeled at the end. For instance, SA-JA(3) will mean the plant received three doses of JA after the initial SA dose.

Staining for ROS

Preparation of DAB

DAB solution was prepared by dissolving DAB in sterile H_2O (1mg:1ml). The mixture was reduced to a pH of 3 to maximize the quantity of DAB dissolved. Following this, the container holding the solution was covered with aluminum foil due to the light sensitivity of the DAB solution. Tween 20 (0.05% v/v) and 200 mM Na_2HPO_4 (5% v/v) were then added to the

solution. Once completed, this generated a 10 mM Na_2HPO_4 DAB staining solution. DAB does not fully dissolve into the solution, yet enough is dissolved to work as an effective stain. The solution is only good for one day, therefore, fresh solution was made for each test (Daudi *et al*., 2012).

Preparation of NBT

A 6mM NBT stain was prepared in sodium citrate at a pH of 6. NBT is light sensitive and so was stored in a container covered with aluminum foil at all times. NBT was also be made fresh for each use (Kaur *et al.*, 2016).

Staining Leaf Tissue with DAB or NBT

Maize, cv. Golden Bantam, seeds were sown under normal conditions in a greenhouse. Leaf tissue was removed from the plant after it has formed a ligule and soaked in water for 12-24 hours. This process allows ROS build up near cut sites to dissipate. Small leaf sections were placed into wells of a 24-well plate. Staining solutions were added to each well to cover leaf tissue (approx. 2ml). Control tissue for DAB experiments received 200 mM Na_2HPO_4. NBT tests control tissue received a sodium citrate solution. The plate was then placed in a desiccator and vacuum infiltrated for 10 minutes to allow for uptake of the stain by the tissue. The vacuum was covered to avoid light from degrading DAB or NBT stain. After removed from the vacuum, the plate itself was covered with aluminum foil. The covered plate was then placed on a shaker at 100 rpm for 4 hours to incubate the tissue in the stain. Following incubation, the stains were replaced with bleaching solution (ethanol: acetic acid: glycerol = 3:1:1). The uncovered plate was then place gently into a water bath (95-100 °C) for 15-20 minutes or until the tissue was

void of chlorophyll. The brown or blue precipitate formed by DAB reacting with H_2O_2 or NBT reacting with $O_2^{\cdot -}$, respectively, will remain in the leaf tissue. Once the chlorophyll was removed from the tissue, the plate was removed from the water bath and new bleaching solution was added. At this stage, tissue was stored in 4°C for up to 4 days in the plate covered with aluminum foil. Leaf tissue was examined using a dissecting microscope. (Daudi *et al.* 2012).

Quantification of Light Absorption Molecules After GYY4137 Application

Corn, cv. Golden Bantam, seeds were planted in potting mix, one seed per pot (6-inches in diameter). They were germinated in the greenhouse and after germination pots were moved outside for the remainder of the experiment. Dosing of GYY4137 started immediately after all the seeds had germinated. The dosing occurred once every week for four weeks. This totaled four treatments of each concentration to the plants. To dose the plants, we used an apparatus that injects 10mL of a solution into the potting mix with each application. Every 10mL dose contained 10mg, 100mg, 200mg, 400mg, or 800mg of GYY4137 dissolved in water. The control plants received only water.

Leaf samples were collected periodically for measurements of chlorophyll content. To do this, 50mg samples from the tip of each leaf were collected and taken through the chlorophyll extraction process (below). I sampled leaves as soon as they were fully expanded This was done for leaves 1 through 6. An error was made with leaf 2 causing the wind to blow away the leaves, resulting in no leaf 2 data.

The chlorophyll extraction was performed by soaking the leaf tip in a chloroform bath for 30 seconds to remove the waxy cuticle and then moving the leaf to a water bath to be rinsed. Once dry, the leaf was placed into a test tube with 3.5mL of DMSO and incubated at 60 degrees

C for 6 hours. 2mL of each extract was pipetted into a well in a 24-well plate. The plates were measured in a SPECTRAmax M2 spectrometer and absorbance readings at 480nm, 649nm, and 665nm were recorded. Absorbance readings were then used in previously formulated equations to quantify chlorophyll a and chlorophyll b and carotenoid content. The equations for DMSO chlorophyll extractions are $Chl_a = 12.19A_{665} - 3.45A_{649}$ and $Chl_b = 21.99A_{649} - 5.32A_{665}$ (Tait and Hik, 2003). The equation for carotenoids is $C = (1000A_{480} - 1.29Chl_a - 53.78Chl_b)/220$ (Nayek et al, 2014).

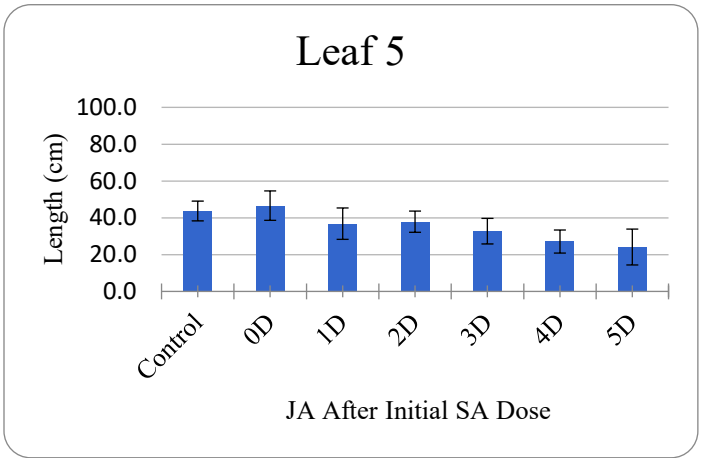
In addition, we conducted a follow-up experiment to test whether GYY4137 alleviated the effect of water stress. To do this, we simply stopped watering the plants for two weeks and recorded which survived the best qualitatively.

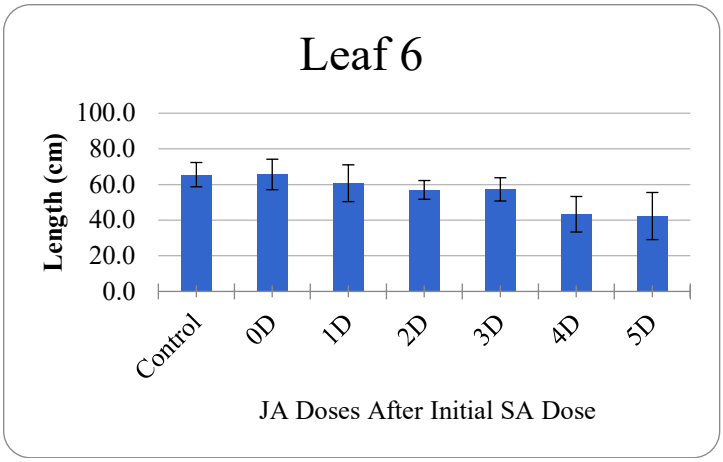
Results

Synergistic Effects of JA and SA

Increasing SA-JA Treatments Shortens Transition Leaves

The lengths of leaves 5 through 9 were recorded and then averaged for each SA-JA dose group and control group. For all the SA-JA dose groups there was a notable length decrease in leaves 5, 6, and 7. The SA-JA(0) group did not show a difference in length from the control. The rest of the SA-JA groups decreased in the leaf length as the doses of JA increased (Figure 1a, 1b, and 1c). By SA-JA(5), leaves 5, 6, and 7 were averaging 20.1 cm shorter than the control. In leaf 5, the control was 43.7 cm long and SA-JA(5) was 24.3 cm long (55.6%the length of the control). The leaf 6 control had a length of 65.5 cm, while the SA-JA(5) group was 42.3 cm long (64.6% the length of the control). The control for leaf 7 had a length of 79.2 cm, whereas the SA-JA(5) was at 61.4 cm long (77.5% the length of the control). Leaves 8 and 9 did not show any large differences in length between the control and plants dosed with SA-JA treatments (Figures 1d and 1e).





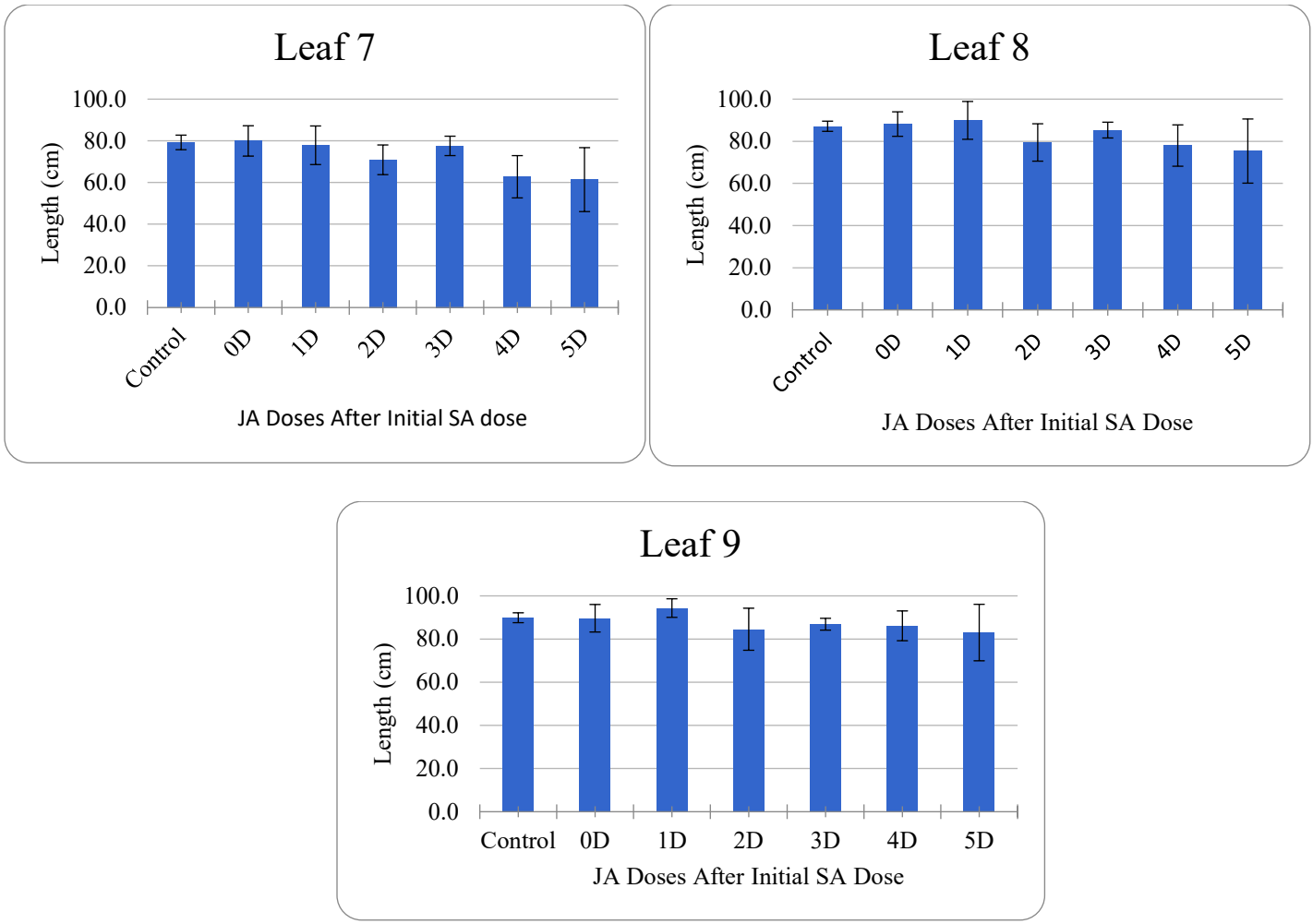


Figure 1: Observing synergistic effects of an initial SA dose followed by varying number of JA doses on length (cm) of the maize leaf.

Length of JA-SA Plants Show Inconsistencies

Leaf length of JA-SA plants were inconsistent over increasing doses, but had a general trend of being shorter than the control. Leaves 5 through 7 showed the most differences and leaves 8 and 9 did not show great difference from the control. The shortest leaves were always on the JA-SA(5) groups. The 5 dose levels for leave 5 through 7 averaged 31.7 cm shorter than the control. Leaf 5 experienced the most inconsistency of any of the leaves. The control was 74.4

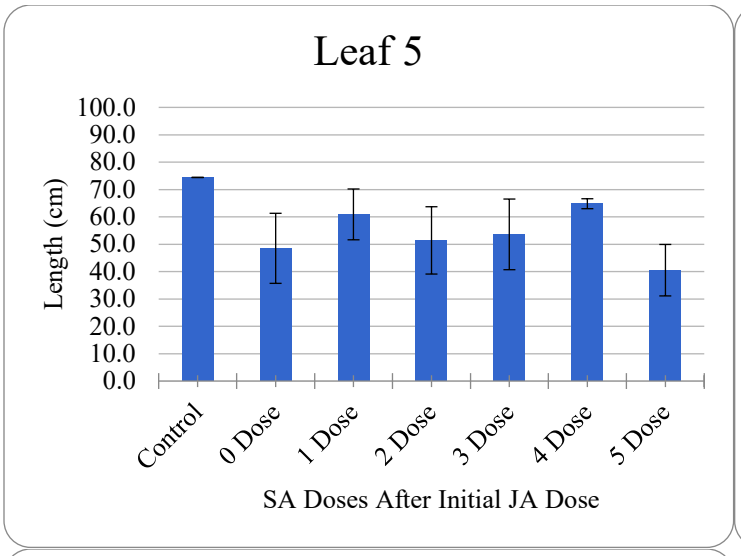
cm long, while JA-SA(0) decreased to 48.5 cm, making it the second shortest of the dosing levels. The next four increasing doses had leaves from 51.4 cm to 64.8 cm. The shortest leaf came at JA-SA(5) with a length of 40.5 cm.

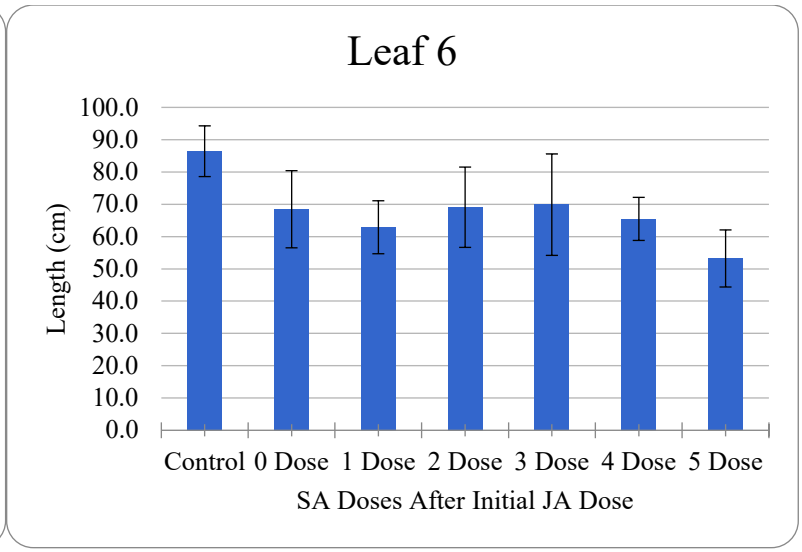
Doses on leaf 6 had a more stable trend than leaf 5. The control was the longest at 86.4 cm. Lengths decreased in JA-SA(0) and JA-SA(1) at lengths of 68.5 cm and 62.9 cm. The next two increasing dosing levels both had increases in leaf length. JA-SA(2) and JA-SA(3) had lengths of 69.1 cm and 69.9 cm. Decreases were seen in the final two levels of 4 and 5. JA-SA(4) had a length of 65.5 cm. JA-SA(5) had the shortest leaf 6 at 53.2 cm.

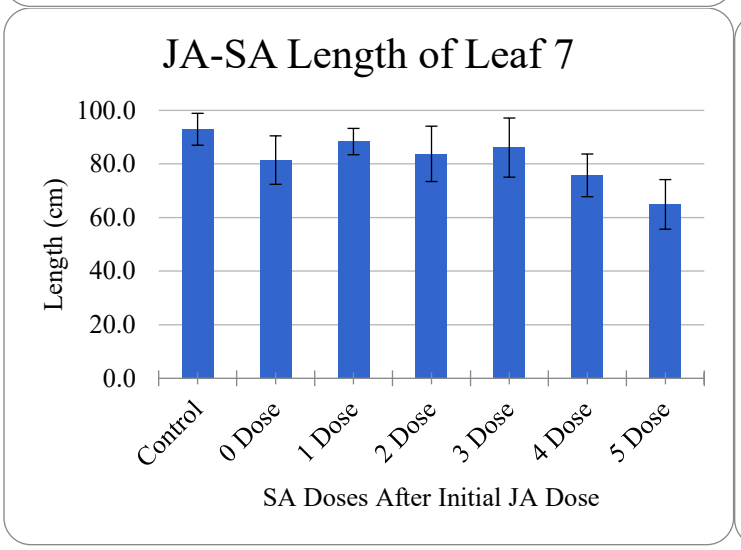
Leaf 7 had a similar trend to leaf 6. The control had a length of 93.0 cm and the JA-SA(0) decreased to 81.5 cm. Doses1 through 3 had very similar lengths ranging from 83.8 cm to 88.4 cm. The next two dose levels showed a steady decrease. Plants JA-SA(4) and JA-SA(5) had lengths of 75.8 cm and 64.9 cm.

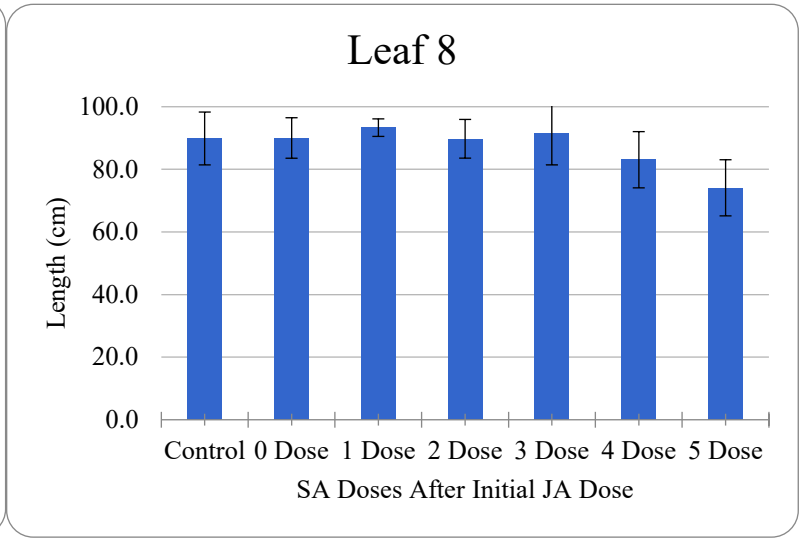
Leaf 8 showed the least amount of difference between dose levels. The control was 89.9 cm long. Doses 0 to 3 had no large difference in length. There was only a difference of plus or minus 3.4 cm. The final two dose levels had a steady decrease like leaf 7. JA-SA(4) had a length of 83.1 cm and JA-SA(5) decreased to the lowest length of leaf 8 at 74.1 cm.

Although leaf 5 was the most inconsistent, leaf 9 had the most abnormal results. The control for leaf 9 was the third shortest at 77.7 cm instead of being the longest. Doses 0 through 3 were all longer than the control in a range of 82.9 cm to 86.9 cm. Yet, similar to the past two leaves, JA-SA(4) and JA-SA(5) on leaf 9 decreased as the doses increased. JA-SA(4) was 75.8 cm and JA-SA(5) was 69.4 cm.









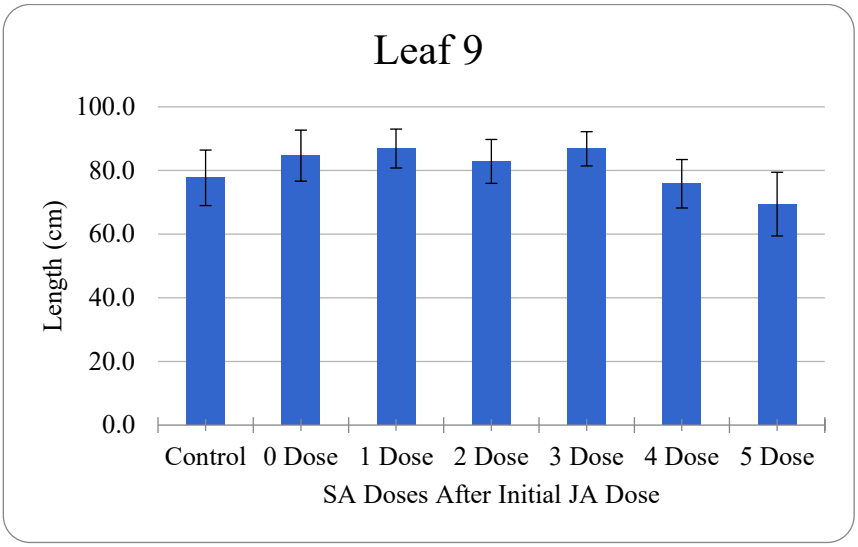


Figure 2: Observing synergistic effects of an initial JA dose followed by varying number of SA doses on length (cm) of the maize leaf.

Examination of Hair Pattern in SA-JA Dosed Maize Shows General Trend of Delay in the Timing of Vegetative Phase Change

The timing of vegetative phase change in leaf 6 among experimental groups was inconsistent, but from the control to SA-JA(4), there was a general delay in the timing of phase change shown by the area of leaf with wax. Leaf 6 of the control group averages 64% of the leaf with waxy epidermis while the SA-JA(4) group had 91%. Dose groups SA-JA(0), SA-JA(1), SA-JA(2), and SA-JA(3) were 86%, 68%, 80%, and 83%, respectively. SA-JA(5) group had a 73% waxy epidermis area, which was inconsistent with the rest of the data.

In leaf 7, we saw a general increase in the area of leaf with waxy epidermis and corresponding decrease in the area of the plant with full hair patterning. The control group had the largest area with full hair patterning (55%) and SA-JA(3) group had the smallest area with full hair patterning (3%). Full hair pattern increased in SA-JA(4) group (8%) and the SA-JA(5) group (28%), but full hair pattern area for these conditions was still less than the control group.

For leaf 8, the trend for timing of vegetative phase change becomes more consistent. The full hair on the control was the largest at 72%. The general trend of the previous leaves continued with the SA-JA(0) group decreasing in full hair (63%) and an increase in the SA-JA(1) group (65%). Each increasing dose grouping had decreasing amounts of full hair with SA-JA(5) group having the smallest area of leaf with full hair pattern (35%).

The trend from the previous leaves continued into leaf 9. The full hair on the control was 79% of the leaf's length. At SA-JA(0), the percentage decreased to 76%, but exactly like the other leaves the SA-JA(1) group had an increase in percentage of the leaf covered in hair (81%).

This is inconsistent with the other leaves because the area of leaf with full hair is greater on the SA-JA(1) group (81%) than the control (79%). All other dose groups followed the same trend as leaf 8 with a steady decrease in the full hair region of the leaf. By SA-JA(5), leaf 8 had 59% of the blade with full hair pattern.

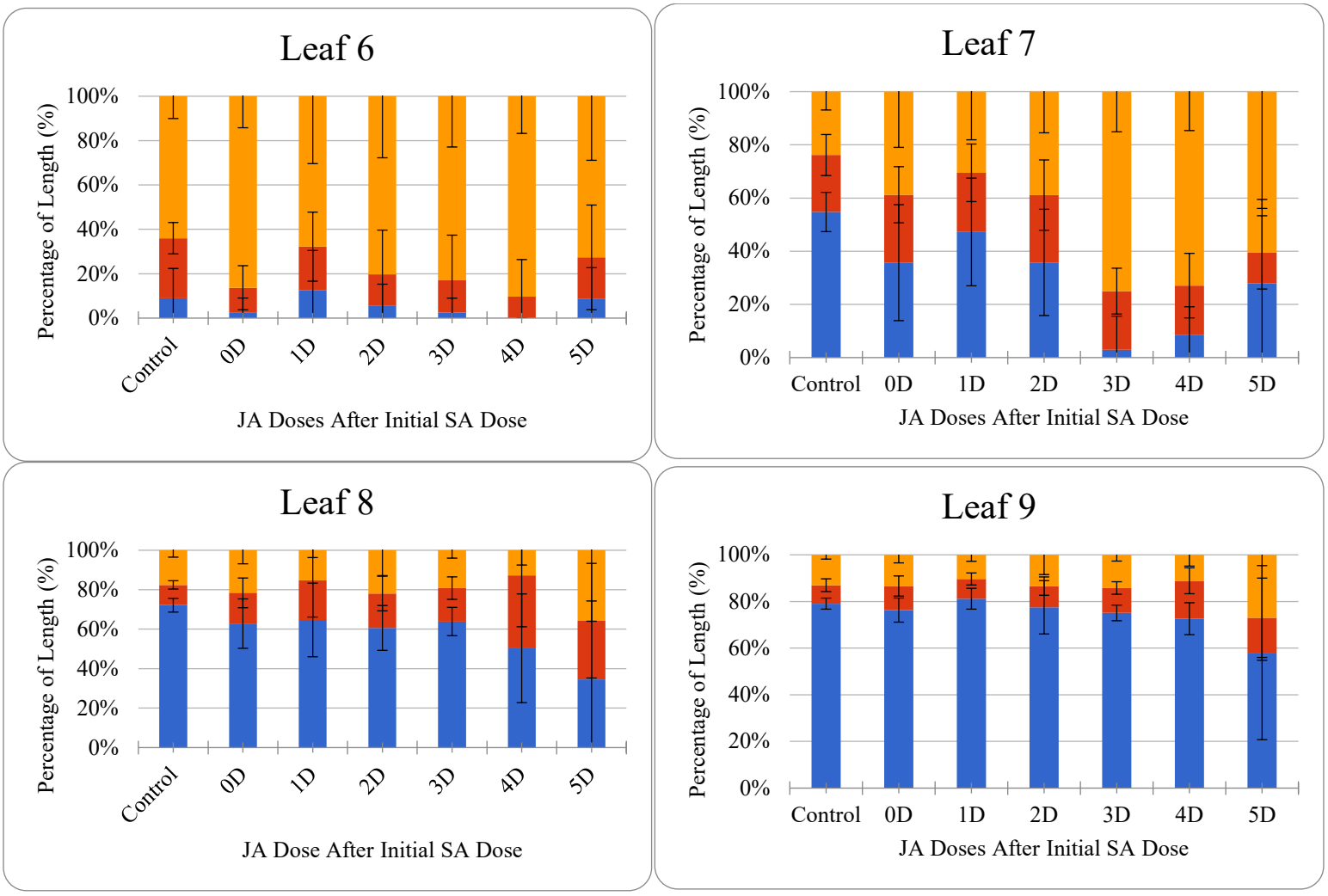


Figure 3: Plants were initially treated with a single dose of SA followed by various number of treatments of JA. Leaves were scored for regions of the leaf expressing hair (blue) and waxy (yellow). Transition regions are shown with red.

Initial JA Dose Causes More Juvenile Hair Pattern Traits, Yet Increasing SA Doses Show No Apparent Effect

The hair pattern over leaves 5 to 9 did not show major differences in the levels that were dosed. However, they all were in a more juvenile state than the control. This was most likely due to the initial dose of JA. On leaf 5 the control had hair on 39% of the leaf. The most hair on any of the dosing was on JA-SA(0), having only 10% of the blade covered in hair. The rest of the doses had no more than 4% of the leaf with hair.

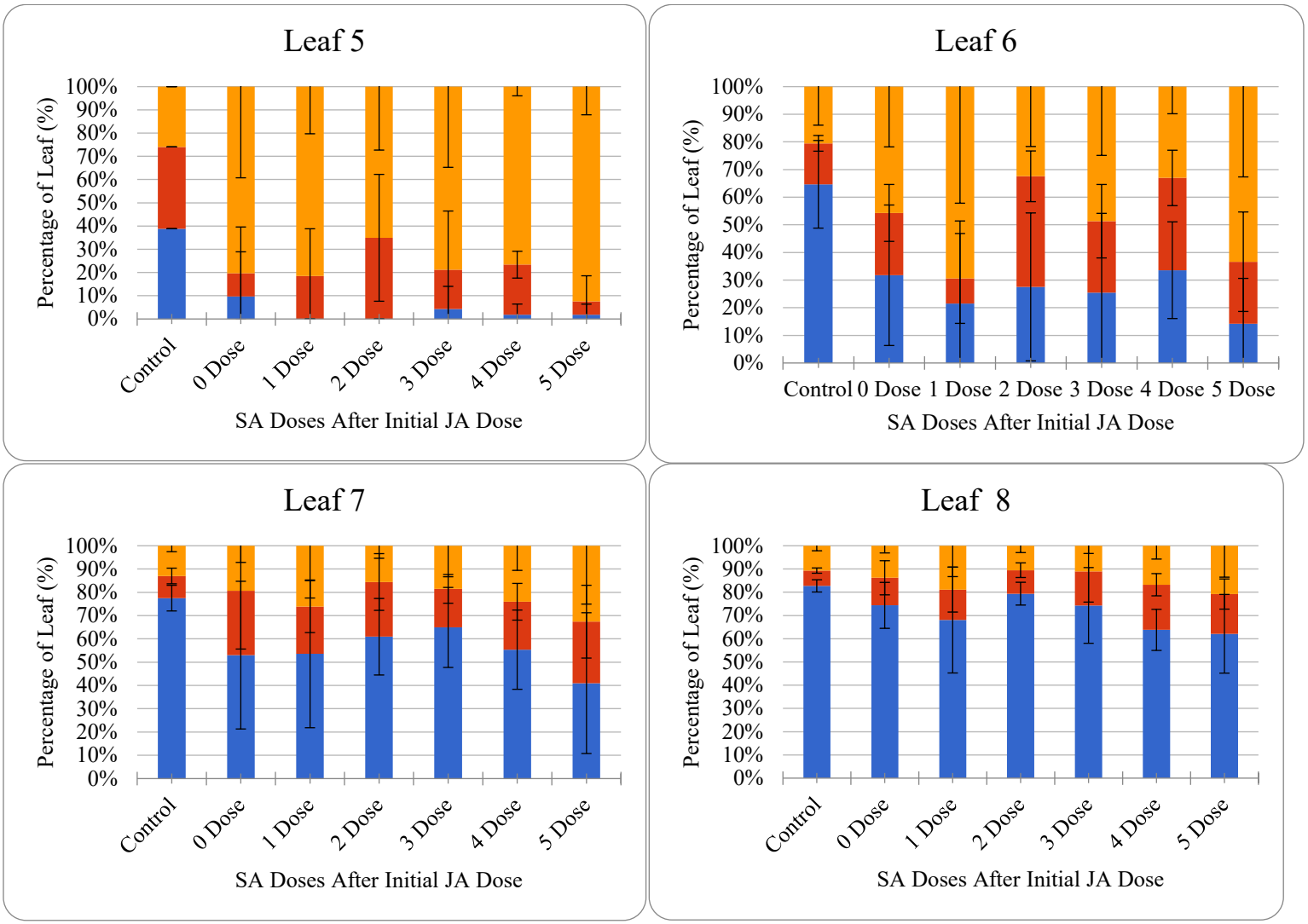
Leaf 6 had higher and more inconsistent percentages of hair, yet they will still relatively similar through all the dosing levels. The control had the most hair on the leaf (65%). JA-SA(0) and JA-SA(4) had the next two highest percentages of hair at 32% and 34%, respectively. Doses 1 (21%), 2 (28%), and 3 (25%) had similar results of hair on their leaf 6. The lowest amount of hair was found on JA-SA(5) with hair on 14% of the blade.

The control for leaf 7 had hair on 78% of the leaf making in the most of all the doses. JA-SA(0) and JA-SA(1) were almost identical with hair on 53% and 54% of the leaf. There is an increase in the next two dosing levels. JA-SA(2) increased to 61% and JA-SA(3) increased even more to 65%. A steady decrease was observed as dosing level increased. JA-SA(4) was at 55% and JA-SA(5) held the low again having hair on 41% of the leaf. Overall, the 0 dose to the 4 dose only ranged from 53% to 65%.

Leaf 8's control had the most amount of hair with hair covering 86% of the blade. Once again, the results for the dosing levels look inconsistent, but still similar to all others. JA-SA(0) had hair on 74% of the leaf and decreased to 68% in JA-SA(1). Oddly, JA-SA(2) had an increase in hair to cover 79% of the leaf. The next three increasing levels showed a larger decrease for

each level. JA-SA(3) was at 74%, JA-SA(4) decreased to 64%, and JA-SA(5) decrease to 62%, the lowest on leaf 8.

Leaf 9 did not have results that differed greatly between the dosing level. The control had 86% of the leaf with hair. This was the largest recorded, but the rest of the levels are ranged from 72% to 82%. Another difference observed on leaf 9 was the two lowest amount of hair were found on JA-SA(2), 72%, and JA-SA(4), 73%.



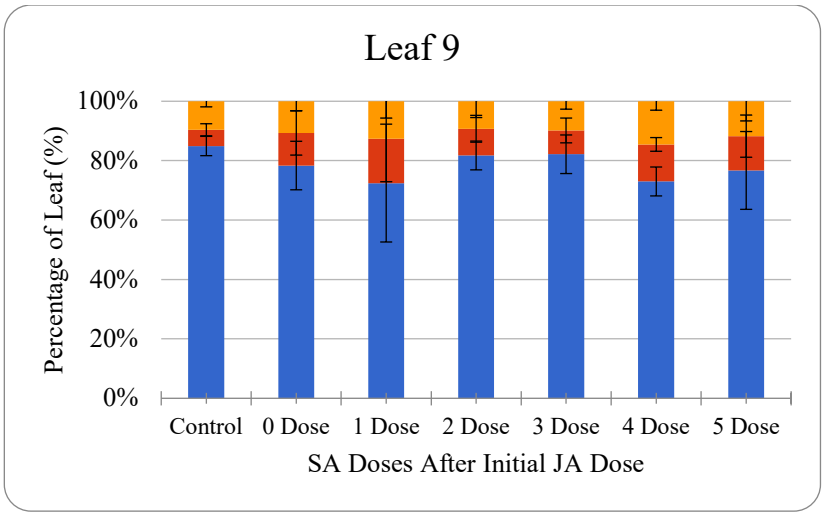


Figure 4: Plants were initially treated with a single dose of JA followed by various number of treatments of SA. Leaves were scored for regions of the leaf expressing hair (blue) and waxy (yellow). Transition regions are shown with red.

Staining with Toluidine Blue Shows Timing of Vegetative Phase Change is Increasingly Delayed with Increasing Doses of SA-JA

Staining for lignin allowed for another way to look at the timing of vegetative phase change by recording the presence, or lack of, lignin in the leaf epidermis cell wall. Lignin is an important trait in the adult phase of maize to create rigidity in the plant and is stained turquoise by toluidine blue. If lignin is not present, the cell walls stain purple. In leaf 6, a trend was similar to what was seen in the hair pattern. The control group had the smallest percentage of the leaf not containing lignin (67%). The SA-JA(0) group decreased to 97% and the SA-JA(1) group increased to 90%. The lack of lignin in the leaf increased as the JA doses increased to the highest value in SA-JA(4), (98%). SA-JA(5)had a decrease in lack of lignin with a value of 95%.

The results for leaf 7 started a different trend than seen previously. Here, the control grouping had no lignin present in 76% of the leaf. In SA-JA(0), the lack of lignin increased to

83%. A decrease in the area lacking lignin occurred in both the SA-JA(1) group (65%) and the SA-JA(2) group (52%). These are unexpected results due to both having more lignin present than the control. SA-JA(3)and SA-JA(4) had an increased area lacking lignin at 83% and 95%, respectively. Then similar to the trend hair pattern had in leaf 7, the SA-JA(5) group decreased in lack of lignin (88%).

In leaf 8, the control group had the longest region of lignin present of the groupings at 40%. There was a decrease in lignin for the SA-JA(0) grouping to 29% and this decrease continued into the SA-JA(4) plants (27%). Unexpectedly, the SA-JA(2) group increased the amount of lignin to 37% of the leaf. SA-JA(3) and SA-JA(4) decreased in lignin to 48% and 45 %, respectively. The SA-JA(5) plants acted the same as the previous leaves and had lignin increase to 23%.

The general trend in staining continued in leaf 9. The control plants had lignin present in 60% of the leaf. The SA-JA(0) and SA-JA(1) groupings both decreased in the amount of lignin at 57% and 48%. Following the trend of staining from the previous leaves, the SA-JA(2) plants had an increased to the highest in the amount of lignin in leaf 9 (61%). SA-JA(3), SA-JA(4), and 5 SA-JA(4) groupings all decreased in amount of lignin at 48%, 45% and 44%, respectively.

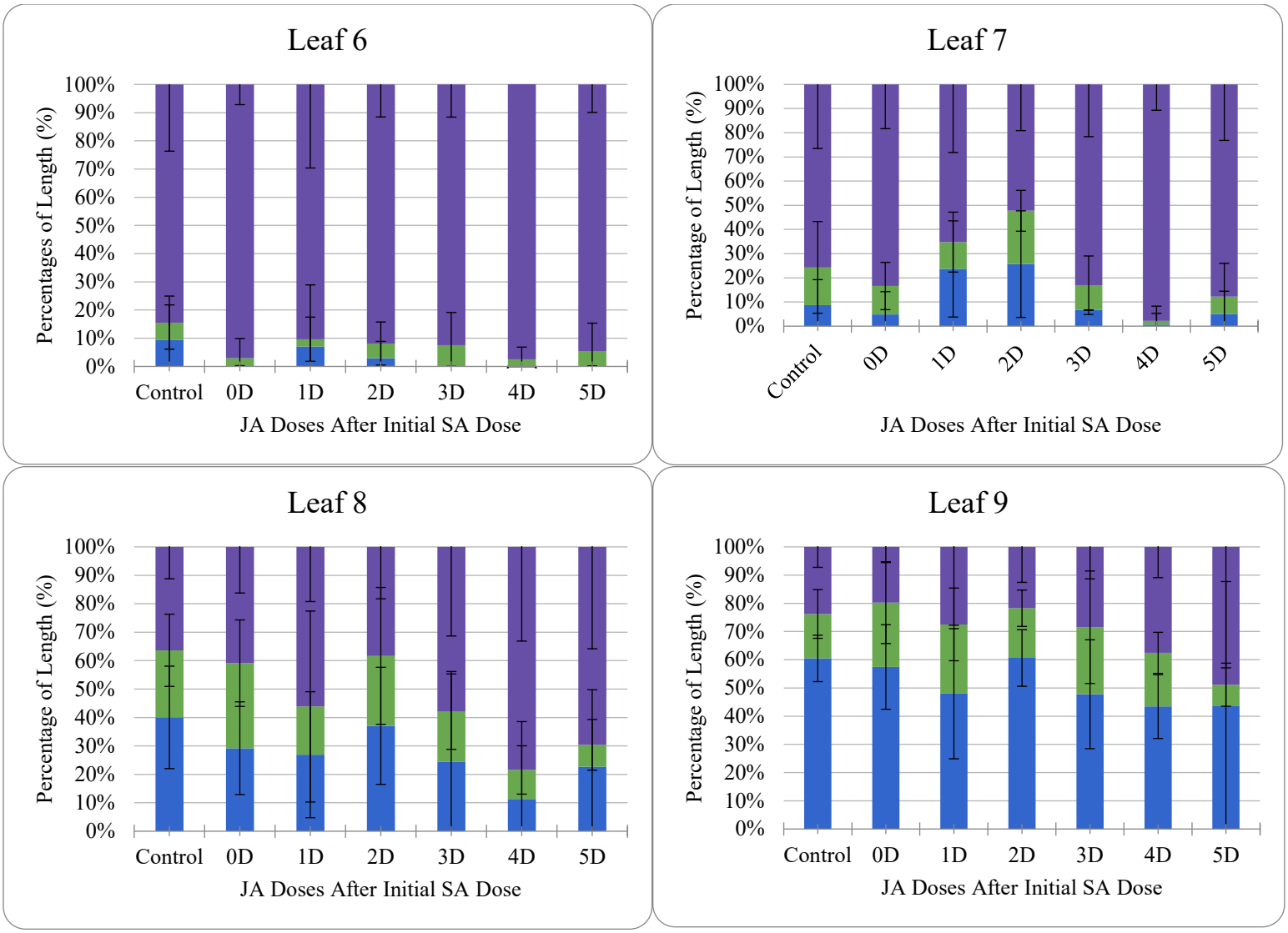


Figure 5: Plants were initially treated with a single dose of SA followed by various number of treatments of JA. Staining with Toluidine Blue of shows whether lignin (blue) is present or absent (purple) in cells of the leaf. A mosaic (green) region is found between these regions.

Initial JA Dose Causes More Juvenile Staining Pattern Traits, Yet Increasing SA Doses Show No Apparent Effect

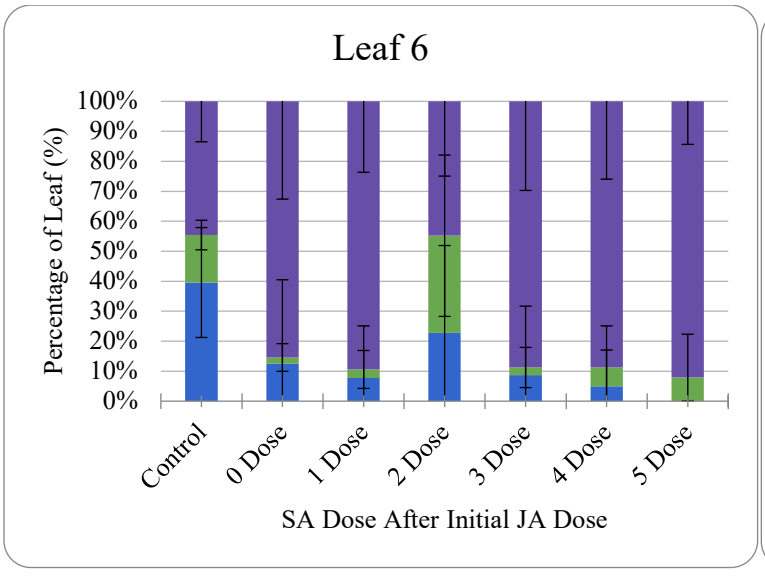
The results of staining in the JA-SA plants showed very similar results to the hair patterning. The control of each leaf had the most region containing lignin and all JA-SA(5) plants always had the least amount of lignin. All leaves also had an inconsistency for JA-SA(2)

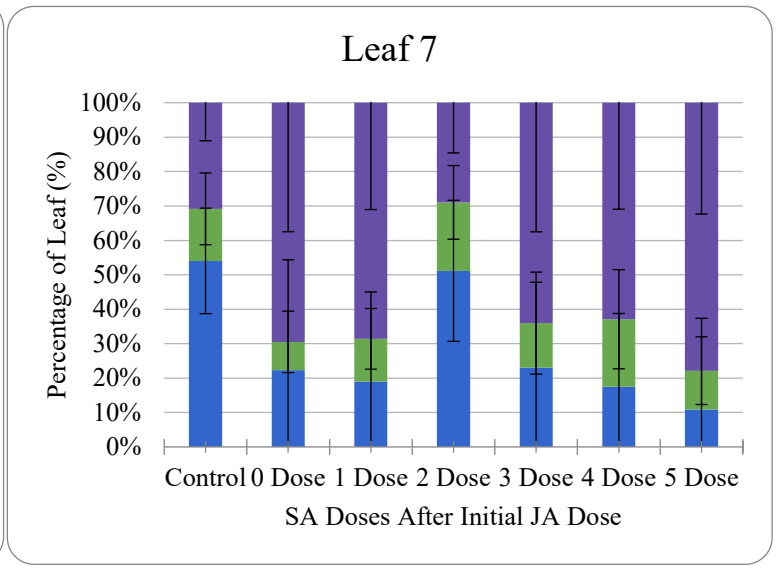
plants. Leaf 6 showed the most severe instance of this event. The control for leaf 6 had lignin in 40% of the leaf. Then, besides JA-SA(2), then rest of the doses ranged from 0% to 13% lignin content in the leaf. JA-SA(2) had lignin in 23% of the leaf, which was unexpected.

The lignin content in leaf 7 showed similar results to leaf 6. The control had lignin in 54% of the blade. All other dosing levels, except JA-SA(2), only differed by 12% total with a range of 11% to 23%. Once again, unexpected results occurred in JA-SA(2) with lignin in 51% of the leaf.

For Leaf 8, the control had an abnormality because it did not have the largest region of lignin (51%). The largest was in JA-SA(2) with lignin in 76% of the leaf. All other dosing levels ranged from 37% to 51% lignin content. In this leaf, lignin percentages are becoming closer to the control plants.

In leaf 9, the control had the most amount of lignin filling 74% of the leaf. All other dosing levels, including JA-SA(2), ranged from 50% to 64%, while not showing any trends in the data.





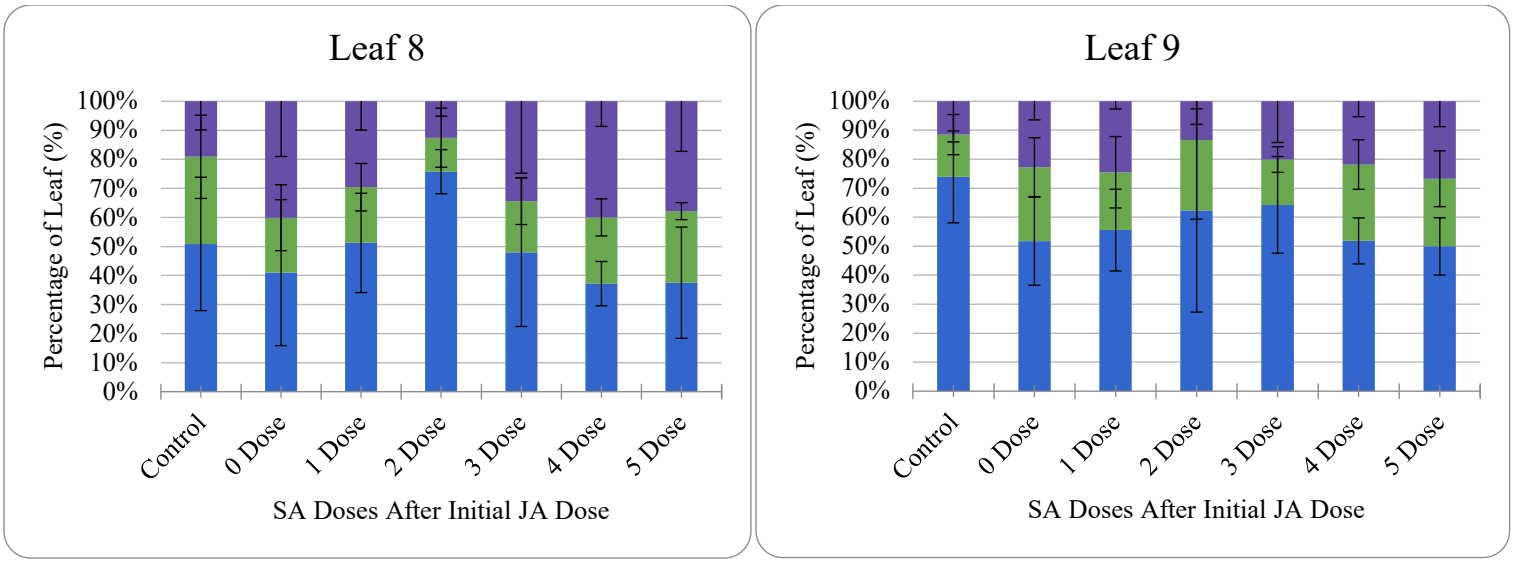


Figure 6: Plants were initially treated with a single dose of JA followed by various number of treatments of SA. Staining with Toluidine Blue of shows whether lignin (blue) is present or absent (purple) in cells of the leaf. A mosaic (green) region is found between these regions.

Protocol for ROS Staining

The results of trial 1 was interesting, but unsuccessful. In Figure 9, we can see that the DAB did stain the leaf tissue in blotches, which appear purple in this image. Trial 1 leaves were observed using a microscope attached to a program that altered the staining color to try and make it more visible. The trial failed because it was difficult to adjust the microscope to obtain the brown stain color of DAB. Furthermore, the initial method of chlorophyll removal was not sufficient to remove all chlorophyll.

In the second trial, I took leaf tissue section from leaf 1 to leaf 6. I also changed to using a dissecting microscope in order to see a wider view of the tissue. I also changed more chlorophyll extraction method to the one stated in the Material and Methods. Figure 10 shows a snapshot of the differences between leaf 1 and leaf 6. From this image, it appears there is more ROS in the form of H_2O_2 in leaf 1 versus leaf 6.

The third trial of perfecting the staining protocol had me soak leaf tissue in water for 12-24 hrs prior to DAB staining to remove ROS build up from removing the leaf from the plant/cutting leaves apart. In Figure 11, I show a comparison of a stained leaf not soaked in water and one that was soaked in water. Here, we can observe that there was much less ROS near the cut site, which allows for us to trust data collected closer to cut sites if needed.

For the fourth trial, I attempted my first round of NBT and DAB. In this trial, I also attempted to stain multiple entire leaf 1's. This method proved to be successful and Figure 12 shows the comparisons of the NBT and DAB stains. There we saw ROS in multiple spot, but was mostly in blotches on the leaf and was unpredictable where the ROS will appear.

Figure 5 shows my fifth and final trial. Here, I attempted to stain entire leaves from leaf 6 to leaf 10. This trial was difficult due to the size of the full leaves. It was difficult to fit them in small enough containers to fit in the vacuum and the water bath to remove the chlorophyll. On top of this, the full size leaves required much more stain, which made it difficult to want to test more than once. In Figure 13, we can see that the chlorophyll was not extracted until the tissue was clear, which makes it difficult to observe the DAB stain. Yet, we can briefly notice that there seems to less ROS in the adult leaves than what we observed in leaf 1.

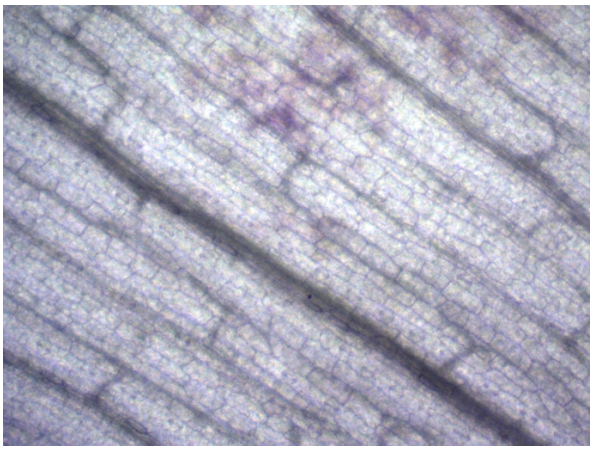


Figure 7: Leaf 1 near the tip stained with DAB. Using other microscope.

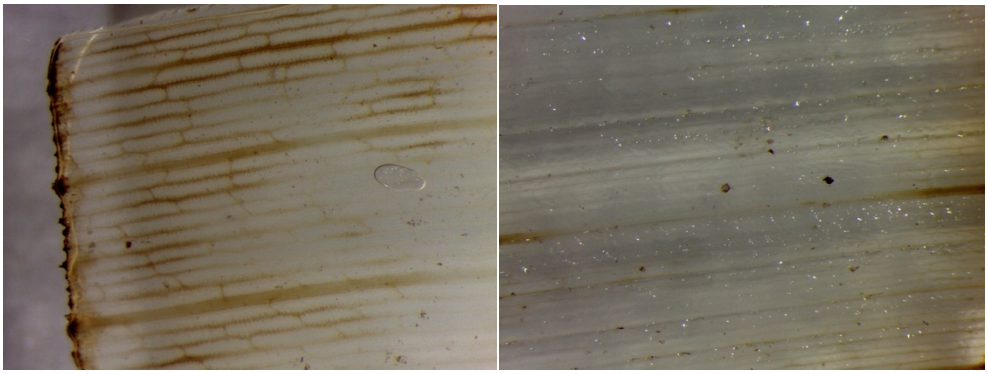


Figure 8: Leaf 1 (left) and leaf 6 (right) comparisons in DAB stain.

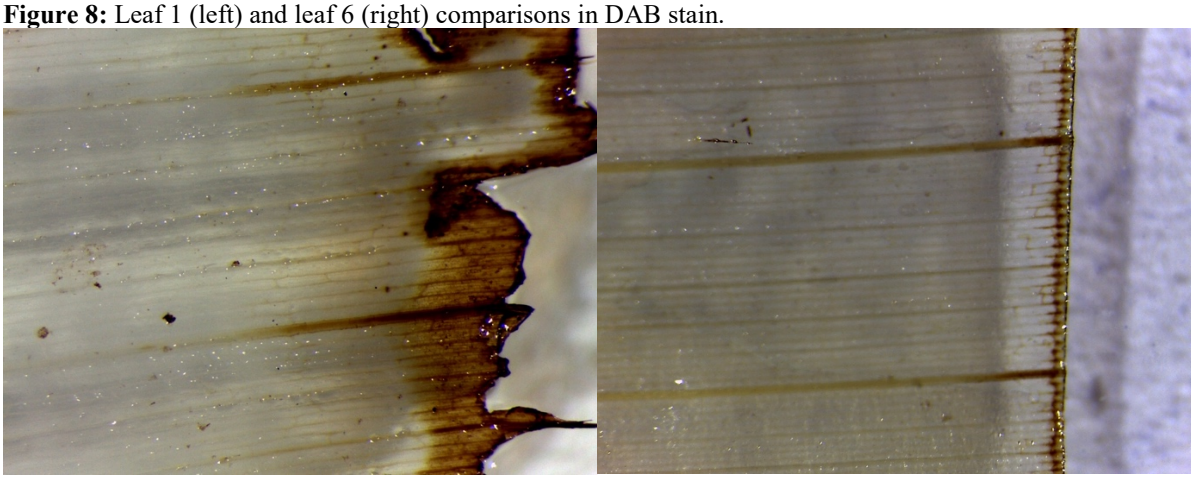


Figure 9: Edge of leaf tissue without (top) and with (bottom) soaking in water prior to DAB staining.

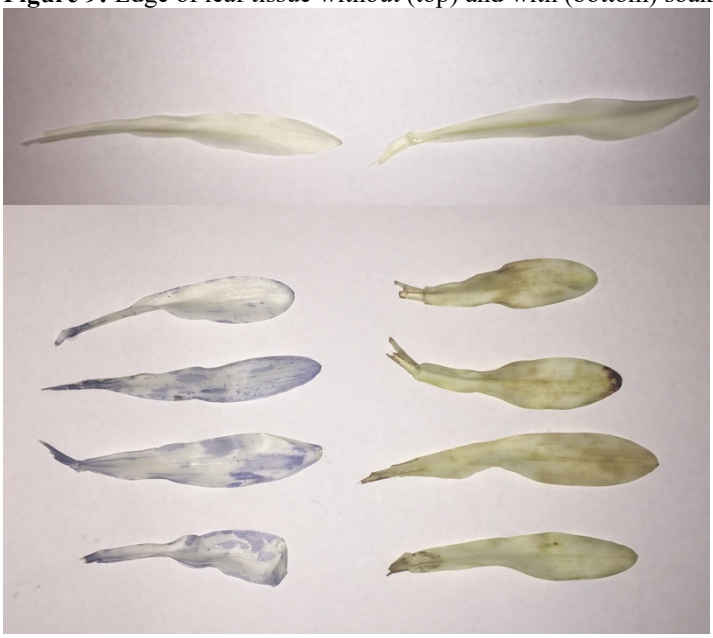


Figure 10: Leaf 1 (juvenile) stained with NBT (left) and DAB (right). A control leaf for each stain is the top leaf for each column.

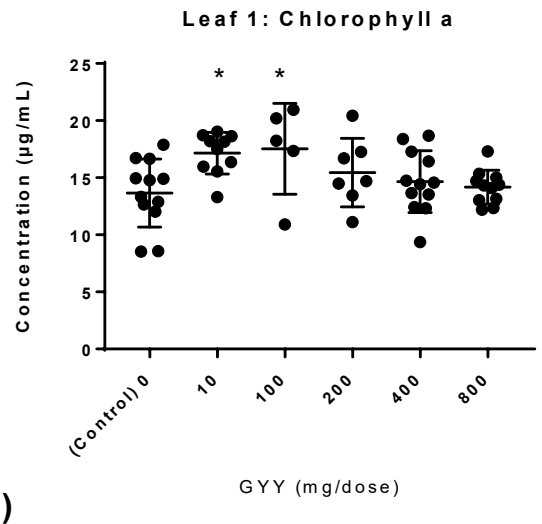
Figure 11: Leaves from 6 (top) to 10 (bottom) stained with DAB.

H_2S as a Signaling Molecule

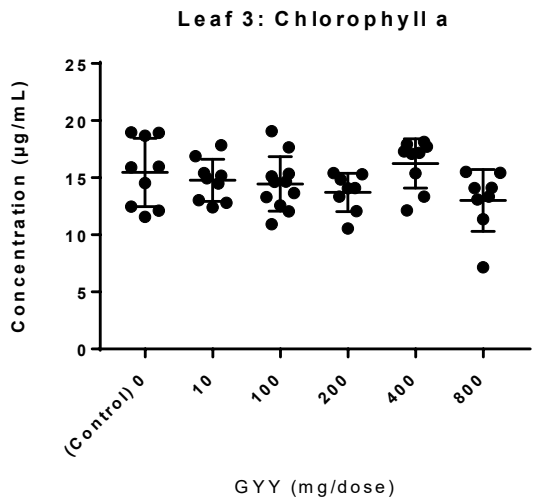
Chlorophyll a Content

Chlorophyll a content of leaf 1 in plants treated with 10mg and 100mg of GYY4137 per dose showed an average increase of 25.6 % (P-Value <.05) and 28.4% from the control, respectively. The increasing doses steadily decreased chlorophyll a content to return close to the control's level by the 800mg dose (Figure 14-A). This difference was also seen in leaves 5 and 6, with an exception of the 100 mg dose in leaf 5 decreased from the value seen in the 10 mg dose (Figure 14-D and E). The 10mg dose in leaf 5 increased 26.7% (P-Value <.05) from the control and the 100mg increased 19.3% (P-Value <.05) from the control. Leaf 3 showed no statistical difference from the control and leaf 4 showed a chlorophyll a content decrease of 5.0% (P-Value <.05) from the control in the 10 mg group. The chlorophyll a content continued to decrease with each sequential higher dose level with the lowest chlorophyll a level experienced at the 800 mg dose, which experienced a decrease of 28.8% (P-Value <.05) from the control (Figure 14-B and C). If looked across the averages of all leaves, it can be seen that the average change is most similar to that experienced by leaves 1, 5, and 6 (Figure 14-F).

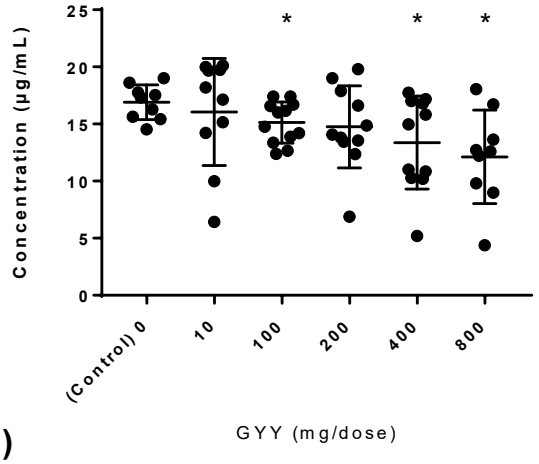
a)


Leaf 1: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

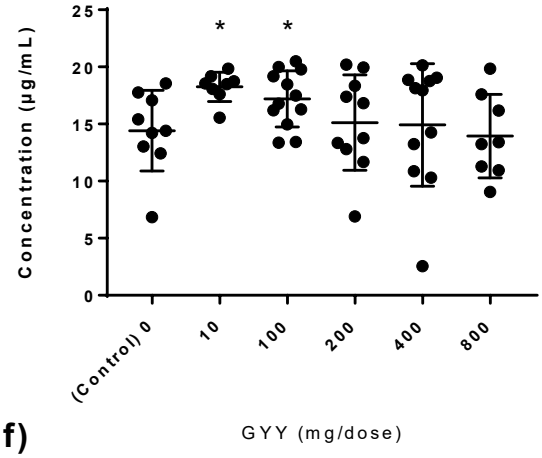
b)


Leaf 3: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
(Control) 0
10
100
200
400
800
GYY (mg/dose)

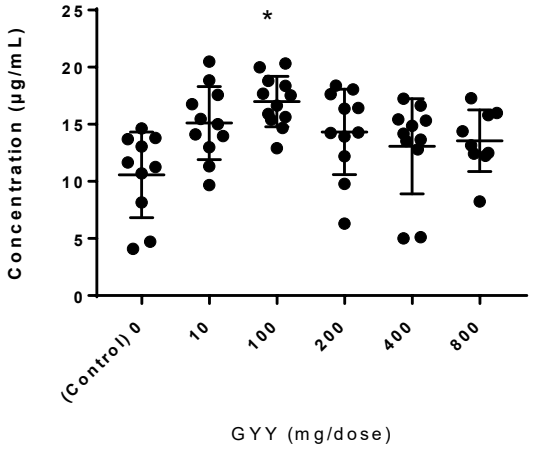
c)


Leaf 4: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
*
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

d)


Leaf 5: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

e)


Leaf 6: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

f)

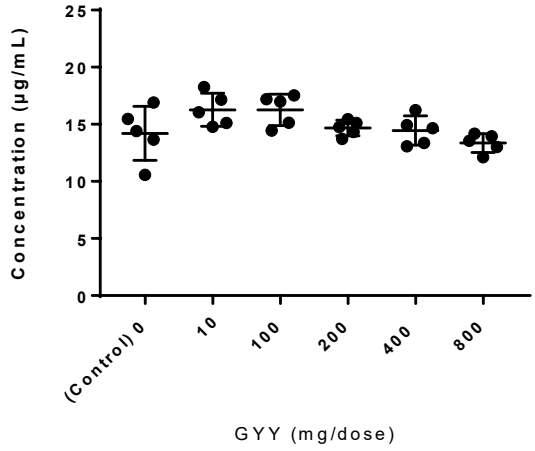
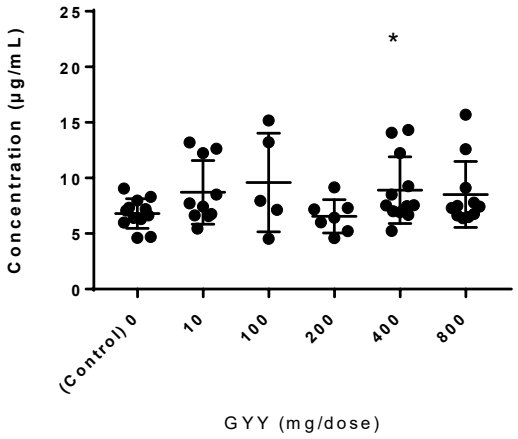

Leaves 1, 3 - 6: Chlorophyll a
Concentration (µg/mL)
25
20
15
10
5
0
(Control) 0
10
100
200
400
800
GYY (mg/dose)

Figure 12: Quantification of chlorophyll a across different doses of GYY4137. Each plant received four total doses over a four-week period. A-E represent each leaf (1, 3-6), respectively. F is a combination of the average for each leaf examined. Each point represents a separate plant tested. Top and bottom lines in the graph are error bars, and the middle line is the mean. Asterisk indicates statistically significant from the control with a P-value <.05.

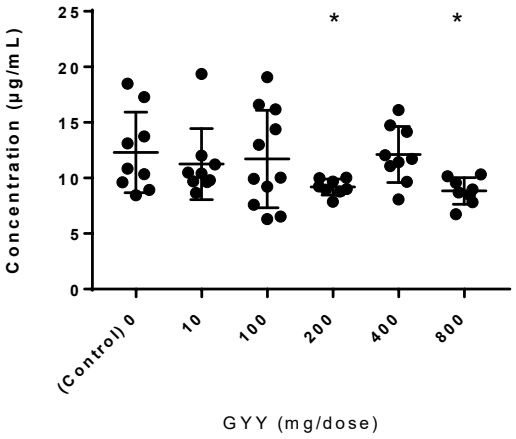
Chlorophyll b Content

The results for chlorophyll b are similar to what was seen in the chlorophyll a results, yet not as pronounced. The only statistically significant data for leaf 1 was in the 400mg group. It had an increase in chlorophyll b of 30.9% from the control (P-value <.05) (Figure 15-A). The other dose averages were all above the average of the control, but were not statistically different. The third leaf had slight statistically significant decreases at the 200mg and 800mg levels. They decreased 25.3% and 28.2% from the control, respectively (P-value <.05) (Figure 15-B). Both leaves 4 and 5 were did not have any treatment levels that were statistically different from the control. (Figure 15-C and D). The final leaf examined (6) showed an increase in chlorophyll b concentration in the leaf for the 10mg and 100mg doses, though only the 100mg treatment level was statistically different from the control. It experienced a 38.1% (P-value <.05) increase from the control. The final three dose levels did not show a significant change from the control (Figure 15-E). Looking at the leaves all together, one could argue that the two smallest dose levels (10mg and 100mg) of GYY4137 was able to increase chlorophyll b slightly, but any greater doses do not show a difference (Figure 15-F).

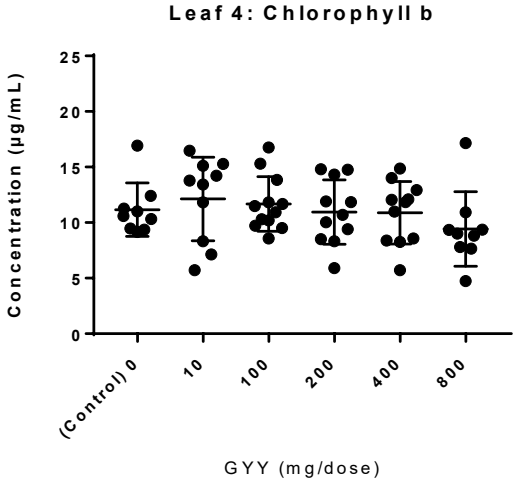
a)


Leaf 1: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

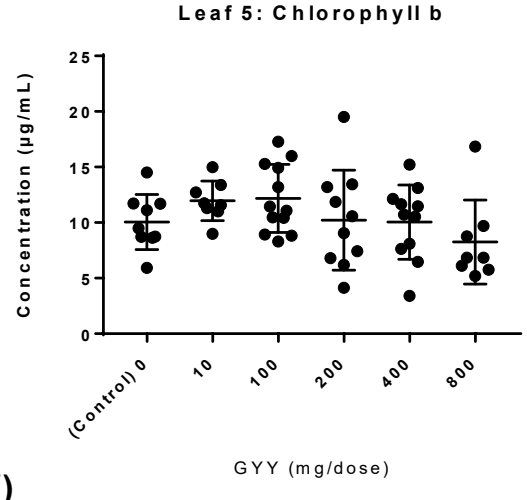
b)


Leaf 3: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

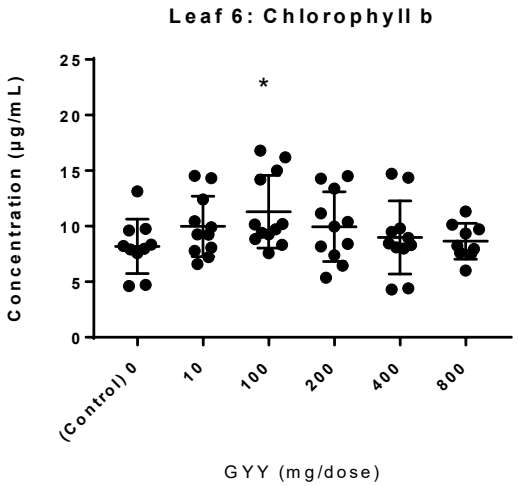
c)


Leaf 4: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
(Control) 0
10
100
200
400
800
GYY (mg/dose)

d)


Leaf 5: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
(Control) 0
10
100
200
400
800
GYY (mg/dose)

e)


Leaf 6: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

f)

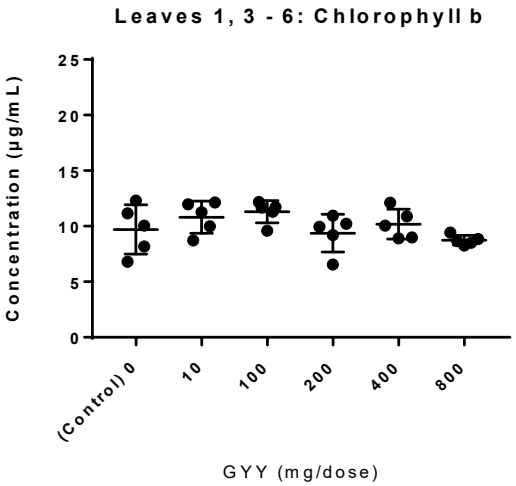
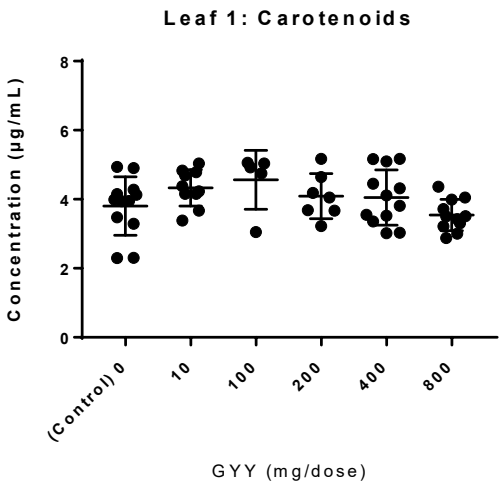

Leaves 1, 3 - 6: Chlorophyll b
Concentration (µg/mL)
25
20
15
10
5
0
(Control) 0
10
100
200
400
800
GYY (mg/dose)

Figure 13: Quantification of chlorophyll b across different doses of GYY4137. Each plant received four total doses over a four-week period. A-E represent each leaf (1, 3-6), respectively. F is a collaboration of the average for each leaf examined. Each point represents a separate plant tested. Top and bottom lines in the graph are error bars, and the middle line is the mean. Asterisk indicates statistically significant from the control with a P-value <.05.

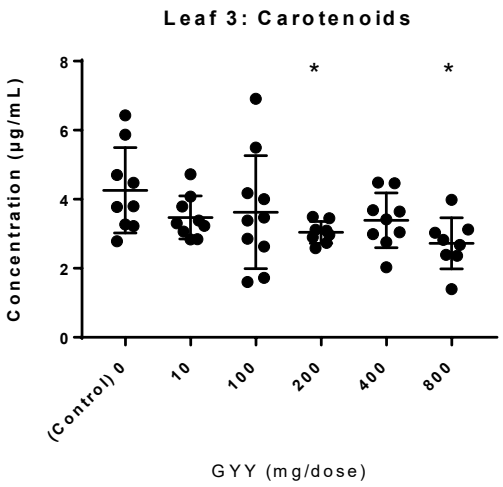
Carotenoid Content

Leaves 1, 5 and 6 did not have any data that was statistically different than the control (Figure 16-A, D, and E). Leaf 3 had a statistically significant decrease in carotenoid content in the leaf at the 200mg and 400mg doses of GYY4137. The 200mg dose decreased 28.6% (P-value <.05) from the control and the 800mg dose decreased 36.1% (P-value <.05) from the control (Figure 16-B). Leaf 4 had decreases in the 100mg, 200mg, 400mg, and 800mg of GYY4137 doses. They all decreased by 43.7%,33.3%, 47.6%, and 51.3% from the control, respectively (P-value <.05) (Figure 16-C). When examining the average of all leaves together, it appears that the control contains the most carotenoids and decreased slightly for each increasing concentration of GYY4137 applied to the plant with the lowest average at the 800mg dose (Figure 16-F).

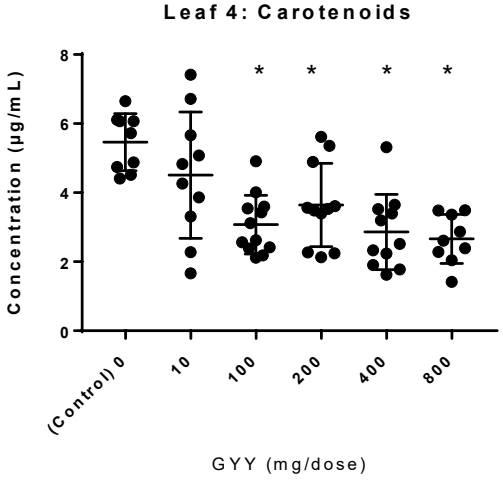
a)


Leaf 1: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
(Control) 0
10
100
200
400
800
GYY (mg/dose)

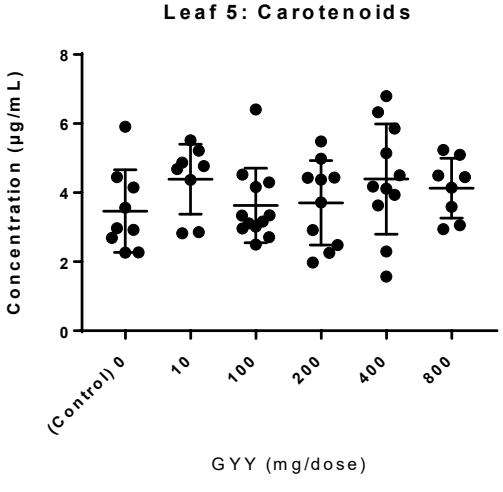
b)


Leaf 3: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

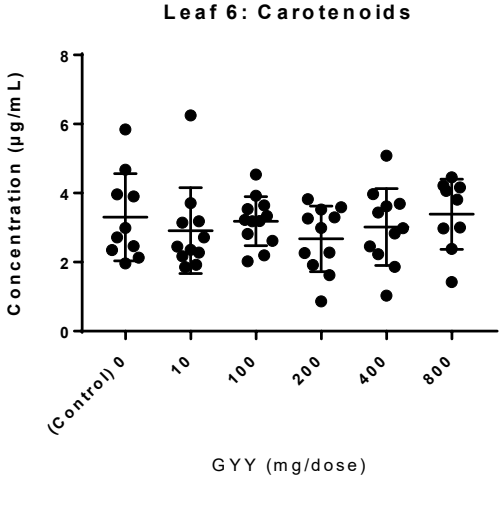
c)


Leaf 4: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
*
*
*
*
(Control) 0
10
100
200
400
800
GYY (mg/dose)

d)


Leaf 5: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
(Control) 0
10
100
200
400
800
GYY (mg/dose)

e)


Leaf 6: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
(Control) 0
10
100
200
400
800
GYY (mg/dose)

f)

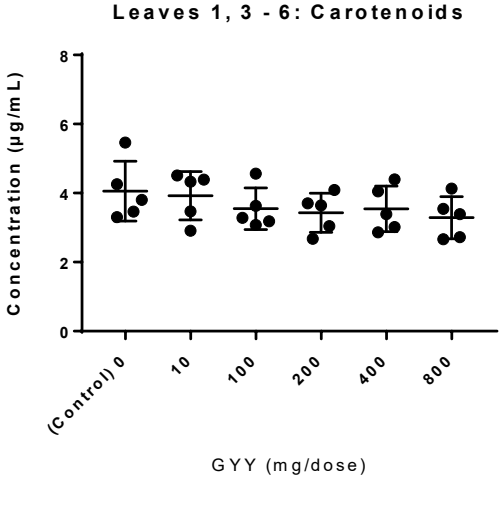

Leaves 1, 3 - 6: Carotenoids
Concentration (µg/mL)
0
2
4
6
8
(Control) 0
10
100
200
400
800
GYY (mg/dose)

Figure 14: Quantification of carotenoids across different doses of GYY4137. Each plant received four total doses over a four-week period. A-E represent each leaf (1, 3-6), respectively. F is a collaboration of the average for each leaf examined. Each point represents a separate plant tested. Top and bottom lines in the graph are error bars, and the middle line is the mean. Asterisk indicates statistically significant from the control with a P-value <.05.

Water Stress

Water stress was tested by not watering the plants for 2 weeks after the collection of leaves and testing for chlorophyll content. At the end of the two weeks, the 200mg and 400mg dosed groups looked the heathiest. They were not experiencing wilting and leaves were not shriveling or dying. The unhealthiest were the 10mg and 100mg of GYY4137 per dose groupings. Both groups were extremely wilted to the point they were laying down flat. Their leaves were completely dried out or starting to die. The controls were still standing, but leaves were starting to die and some plants were wilting. The abnormality of the plants was experienced in the 800mg plants, which were noticeably shorter than the rest of the groupings including the control. The 800mg group were starting to die at the tips of the leaves and some had a little wilting, but were stable to stand.

Figure 15: Images go in order of increasing GYY4137 dose starting with the Control and ending with the 800mg. Images show the single most average plant of the group.

Discussion

SA-JA Plants Experienced Increased Levels of Juvenile Traits, But Initial SA Dose Leads to Less Change in Vegetative Phase Change Than Expected

By observing the length, hair pattern and staining pattern of all SA-JA plants, we can conclude that there was a greater delay in vegetative phase change as the level of JA dosing increased. The reasoning behind this results from increased juvenile traits that were observed as the dosing levels increased in each of the three experiments. We also believe that the initial SA dose may have resulted in less juvenility than expected from previous experiments in our lab. In the previous experiment, the juvenile traits showed a steadier increase with increasing doses. Therefore, we believe SA had some sort of effect on the experiment. Another difference in the two experiments is timing of dosing. In this paper, doses were given every day, whereas in Beydler et al. 2016, doses were given 2 days apart.

JA-SA Plants Experienced Increased Juvenility From Initial JA Dose, but Increasing SA Doses Lead To No Apparent Effect

Overall, the combination of examining length, hair pattern, and staining pattern concludes that the initial JA dose did cause increased juvenility than observed in the control. In each of the tests all dosing levels showed more juvenile traits except for one instance in staining leaf 8 on JA-SA(2). From there, the following increasing levels of SA doses have shown no effect on vegetative phase change. This can be concluded from the results because no large differences or trends observed in any of the data across dosing levels. Another observation made in both parts

of the experiment was that as the leaf number increased the effect of the hormones became less apparent.

Error in Procedure

In the end, we believe that the inconsistency in the results is due to variability in the brightness of the bulbs in the greenhouse. Therefore, the leaf length could be independent of dosing level, and dependent on the light. Another problem we think occurred when the seedlings were being dosed. Here, it was difficult to ensure all of the plant hormone made it into the apical whorl. At times, the plant could not hold the full dose and some would overflow or fall out. This could have been the reasoning behind such large error that was experienced in the data. Another abnormality experienced was the comparison of the transition zone in hair pattern and the mosaic region in staining. In Beydler et al. 2016, they observed the mosaic region to usually be closer to the tip of the blade than the transition zone. In this experiment, it was the opposite.

Protocol for ROS Staining

In developing this protocol, I think there is some evidence to warrant more testing to discover if in fact there is more ROS in the juvenile leaves than the adult leaves. From observing the leaf 1 NBT and DAB stained leaves and comparing them to leaf 10, we see that there is more ROS stained in leaf 1. I believe further examination will prove our hypothesis. Yet, while continuing this experiment, I would ensure to do leaf 1 in it's entirety, but larger leaves should be done in sections to ensure the protocol is completed as it should be. I could also see this experiment going smoother and more efficiently if a different plant with smaller

leaves was used. For instance, if this was done with *Arabidopsis,* leaves would be able to be placed in a 24 well plate. This would allow for easy vacuum infiltration and chlorophyll extraction, while using less chemicals. Another issue that occurred was I did not receive NBT during the semester, therefore, it was arduous to complete the experiment and collect all the data that I would have liked to.

H_2S as a Signaling Molecule

At low doses of GYY4137, there was statistically significant increase in the amount of chlorophyll a in leaves 1, 5, and 6. Statistically, trends were almost non-existent, therefore if experimental errors were minimized better results could be produced. As such, we believe that this data is not showing the full details of the GYY4137's possible effects. Chlorophyll b and carotenoids did not have a large enough response to GYY4137 to warrant further studies. Currently, we still do not understand the mechanism of H_2S as a signaling molecule. Chen et. al (2011) observed low concentrations of H_2S affected photosynthetic characteristics in *S. oleracea.* Experiments show H_2S may be involved in regulation of photosynthesis, but the mechanism is not understood.

With error occurring early in the experiment due to the learning curve of developing a new process, there could be more consistent data developed. Other errors that may have happened are as follows: the leaves may not have all had equal time to sit in chloroform due to the assembly line process I was trying to maneuver, if the leaf did not sit in the DMSO entirely submerged, which could have been avoided by using more DMSO. Instead DMSO was conserved due to cost and lack of supplies. Another element that should be added is a larger

sample size. Twelve plants were too few for the experiment, especially if a leaf was cut under weight. Another problem that occurred was spider mites infested most of the plants for leaves 4, 5, and 6. Another problem that occurred was we could not get more GYY4137 to execute the fourth and final 800mg dose.

If the project was to be done again, I would start by increasing the sample size to at least 20 plants. Furthermore, this experiment is very time consuming and therefore larger chucks of time are needed to complete it unrushed. I would also like to use more DMSO per leaf to ensure all photosynthetic elements of every leaf were completely extracted. If the experiment were to be run again I would also ensure to get all leaves between 1 and 6 without loosing any, and possibly explore what happens beyond leaf 6 by harvesting more leaves.

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